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(U) Five subjects exercised on a cycle ergometer for 30 min at 55% peak $\dot{V}O_2$ on two occasions in a slightly warm environment (T_a=29°C; T_{dp}=10°C). Pyridostigmine bromide (PYR), an acetylcholinesterase (AChE) inhibitor, was ingested (30 mg) approximately 150 min before one experiment, while the other was a control experiment. Red cell AChE inhibition averaged 40(±7)% during PYR treatment. Esophageal temperature (T_{es}), an 8-site mean skin temperature, forearm blood flow (venous occlusion plethysmography) cutaneous perfusion (laser doppler velocimetry; LDF), and metabolic rate (indirect calorimetry) were measured. Cutaneous perfusion decreased 37% after PYR treatment compared to control ($P \leq 0.05$). During control experiments the T_{es} threshold for initiation of cutaneous perfusion was 36.84(±0.3)°C and increased to 37.03(±0.3)°C with PYR ($P \leq 0.01$). The slope of the LDF:T_{es} relationship was decreased 35% with PYR treatment ($P=0.22$). Forearm blood flow, which included inactive muscle, skin, fat and bone tissue, was not different between treatments, which implies that blood flow to one of those tissues may have increased while skin blood flow decreased during PYR treatment.

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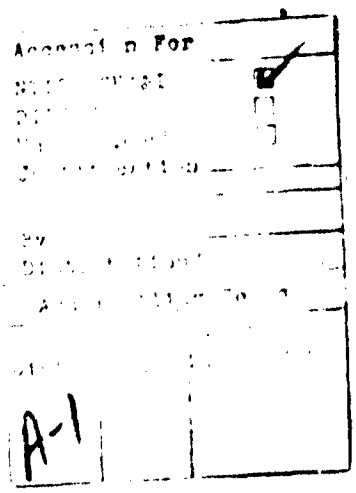
!9. Abstract (cont'd)

The increased threshold for initiation of cutaneous vasodilation with AChE inhibition by PYR suggests that the drug activates central modulation of thermoregulation. One of several possible mechanisms activated may be through increased ACh accumulation of preganglionic sites. This could potentiate adrenergic transmission to cutaneous blood vessels, and enhance vasoconstrictor tone.



ACETYLCHOLINESTERASE INHIBITOR, PYRIDOSTIGMINE BROMIDE, REDUCES SKIN BLOOD FLOW IN HUMANS

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ABSTRACT

Five subjects exercised on a cycle ergometer for 30 min at 55% peak $\dot{V}O_2$ on two occasions in a slightly warm environment (T_a=29°C; T_{dp}=10°C). Pyridostigmine bromide (PYR), an acetylcholinesterase (AChE) inhibitor, was ingested (30 mg) approximately 150 min before one experiment, and no drug was administered during the other experiment (control). Red cell AChE inhibition averaged 40(±7)% during PYR treatment. Esophageal temperature (T_{es}), an 8-site derived mean skin temperature, forearm blood flow (venous occlusion plethysmography), cutaneous perfusion (laser doppler velocimetry; LDF), and metabolic rate (indirect calorimetry) were measured. Cutaneous perfusion decreased 37% after PYR treatment compared to control ($p \leq 0.05$). The T_{es} threshold for initiation of cutaneous perfusion was 36.84(±0.3)°C for the control group and 37.03(±0.3)°C for the PYR group ($p \leq 0.01$). The slope of the LDF:T_{es} relationship was decreased 35% with PYR treatment ($p=0.22$). Forearm blood flow, which included inactive muscle, skin, fat and bone tissue, was not different between treatments, which implies that blood flow to one of those tissues may have increased while skin blood flow decreased during PYR treatment. The increased threshold for initiation of cutaneous vasodilation with AChE inhibition by PYR suggests that the drug activates central modulation of thermoregulation. One of several possible mechanisms activated may be through increased ACh accumulation at preganglionic sites. This could potentiate adrenergic transmission to cutaneous blood vessels, and enhance vasoconstrictor tone.

Index Terms: body temperature regulation, sweating, vasoconstrictor tone, anticholinesterase, pyridostigmine

Sympathetic nervous control of cutaneous blood flow in humans is thought to occur as a dual control system consisting of adrenergic vasoconstrictor fibers (2,25,26) and active vasodilator fibers (8,9,14,24,25,26). During rest in a thermoneutral environment, both vasoconstrictor and vasodilatory fibers to the skin are inactive (2,37). In a cool environment, when skin temperature is cool, cutaneous blood flow is decreased, perhaps as the result of an increase in release of norepinephrine (NE) from the sympathetic nervous system. NE triggers smooth muscle contraction of the blood vessels by acting on α-adrenoreceptors (24). During warming, there is initially a small, passive vasodilation which is thought to occur from release of sympathetic vasoconstrictor activity. Subsequently, a much greater vasodilation occurs (8,26,27), which is thought to be mediated by sympathetic vasodilatory fibers (26,37). The evidence supporting the hypothesis that there are active vasodilatory fibers[1] came from experiments during which the sympathetic nervous system in the arm was blocked. Indirect body heating after sympathetic blockade did not provoke active vasodilation, but did result in passive vasodilation (3,8,14,26,40).

The evidence for the cholinergic nature of the sympathetic vasodilatory fibers to the human skin started to accumulate more than fifty years ago when it was reported that intra-arterial infusion of acetylcholine (ACh) caused the skin of the arm to become intensely flushed (5,7). Intravenous infusion of ACh caused the upper body and face to flush and skin temperature to increase. The effects of ACh infusion subsided several minutes after infusion. Later, mecholyl iontophoresis was used to study the effect of an acetylcholine agonist on human forearm blood flow (1). It was concluded from that study that the acetylcholine

[1] Vasodilatory fiber activity has not been directly measured in human skin nerves, but it has been suggested that efferent sudomotor activity is consonant with reflex vasodilatory activity, at least in the posterior antebrachial nerve (2).

agonist significantly increased forearm blood flow.

Roddie et al. (26) reported that the intra-arterial infusion of atropine, an anticholinergic agent, was associated with a delayed increase in forearm blood flow in response to whole-body heating, and that the magnitude of the vasodilation was reduced as compared to the control arm. It was suggested that this finding was consistent with a cholinergic mechanism of cutaneous vasodilation (26). More recently, the cholinergic nature of active cutaneous vasodilation has been questioned (4,17). However, in vitro experiments on isolated mammalian blood vessels supported the theory that the neurotransmitter which controlled vasodilation of cutaneous vessels with intact endothelium (10) was ACh, because the concentration of ACh perfused was directly related to the degree of relaxation of the isolated blood vessel.

Our experiments (20) on the effect of systemic atropine administration on cutaneous blood flow in exercising men strongly suggested that ACh was not the specific neurotransmitter involved in reflex cutaneous vasodilation. Specifically, systemic atropine administered 30 min before exercise was associated with a lower esophageal temperature threshold for the initiation of cutaneous vasodilation during exercise. The dramatic increase in forearm blood flow after systemic atropine administration indicated that competitive inhibition of ACh had the opposite effect than would be postulated if ACh caused increased forearm blood flow as Roddie reported (26). We suggested that some vasoactive agent other than ACh was responsible for the observed atropine-induced cutaneous vasodilation (20). We further speculated that those data might be explained by the co-neurotransmitter theory of release at the eccrine sweat gland as had been suggested by Lundberg et al. (21) for salivary glands. Also, atropine-induced cutaneous vasodilation may have been achieved via an endothelium-independent mechanism.

The current investigation was done to determine whether inhibition of acetylcholinesterase, and the resultant accumulation of acetylcholine, would affect reflex cutaneous vasodilation in humans during exercise. We report that acute oral treatment with pyridostigmine bromide was associated with decreased skin blood flow as estimated from laser doppler velocimetry.

METHODS

Seven subjects (six males and one female) volunteered to serve as subjects after they were verbally apprised of the nature and risks of the study. However, the critical skin blood flow data was accurately measured in five subjects (four males and one female) and the data from those five subjects are reported here. The physical characteristics (mean ± standard deviation) are as follows: age: 25.8(±6.9) years; height: 1.74(±0.9) m; weight: 72.2(±7.0) kg; body surface area: 1.86(±0.12) m^2; and peak oxygen consumption: 3.38(±0.5) $L \cdot min^{-1}$.

The subjects were completely familiar with all laboratory techniques and practiced those techniques on at least three separate occasions before the study began. Prior to the study, a peak $\dot{V}O_2$ test, consisting of a graded increase in workload every two minutes, was done by each subject during cycle exercise until the cycling rate was not maintained or he/she quit pedalling. Each subject's peak $\dot{V}O_2$ was used to calculate the individual's workload necessary to elicit 55% peak $\dot{V}O_2$.

There were two experiments per subject in this study and both experiments were done at the same time of day in the fall. Subjects were tested twice in an ambient temperature (T_a) of 29°C with an ambient water vapor pressure (P_w) equal to 1 kPa; once 150 min[2] after the

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[2]Peak plasma levels of pyridostigmine bromide and AChE inhibition in humans after ingestion of 30 mg pyridostigmine bromide occur between 2 and 3 hours (23).

ingestion of 30 mg pyridostimine bromide (PYR, Roche, UK, Lot BK94626) and once when no drug was given. Experiments were conducted between 0900 and 1200 h and each subject was studied at the same time of day (33).

Each subject reported to the environmental test chamber dressed in shorts, shoes, and socks for the experiment. (The woman was studied in the follicular phase of her menstrual cycle during which time the control of thermoregulation is most like men (32). She wore shorts, a sleeveless shirt, socks and shoes.) After body weight was determined, each subject sat in a chair which was attached behind a cycle ergometer. A copper-constantan thermocouple encapsulated in a catheter was swallowed for the measurement of esophageal temperature and thermocouples (coppper-constantan) were attached to the skin at eight sites (13,22). Venous occlusion plethysmography (VOP) was used to measure forearm blood flow (FBF) (16,39) and laser doppler velocimetry (LDF) was used as an index of skin blood flow (SkBF) on the forearm (19,30,36).

After all instruments were attached to the subject, a 15 min resting period was initiated. During this period, esophageal and skin temperatures, forearm blood flow and skin blood flow were measured every 30 sec. Metabolic heat production (M) was measured by open circuit spirometry (Sensor Medics) at 7 to 12 min of rest. Exercise at 55% peak $\dot{V}O_2$ began after 15 min of rest and continued for 30 min. All measurements were continued during exercise as described for rest, except that metabolic rate was measured once during exercise at 16 to 20 min.

A 5 ml venous blood sample was drawn before and 150 min after drug treatment and red blood cell acetylcholinesterase (AChE) was determined using a Technicon analyzer (15). The inhibition of AChE in the red blood cell is approximately in parallel with that of the

nerves, muscle and glands and has been justified as an index of tissue inhibition (6).

The T_{es} threshold for cutaneous vasodilation was calculated for each experiment by analyzing the exercise transient phase of the skin blood flow (LDF) to T_{es} relationship. The exercise transient phase is that time of exercise during which T_{es}, skin blood flow and sweating rate rapidly increased. A regression equation was calculated for each subject during the exercise transient for LDF to T_{es}. During the first several min of exercise LDF usually decreased when T_{es} was increasing. These data, and the data after T_{es} reached a steady state, were not included in the linear regression equation. The T_{es} threshold for initiation of cutaneous vasodilation was calculated from the regression equation from the individual's average resting skin blood flow for the control experiment.

Most data were analyzed by a two way analysis of variance with repeated measures, with the two factors being rest/exercise and control/pyridostigmine treatment. The effect of the drug on whole body sweating rate, change in esophageal temperature during exercise, T_{es} threshold for onset of cutaneous vasodilation and slope of the SkBF to T_{es} relationship was analysed using one way analysis of variance with repeated measures. Post hoc tests (Tukeys) were performed when appropriate.

RESULTS

Red cell AChE was inhibited by 40(±7)% at 150 min after pyridostigmine bromide ingestion. Metabolic rate was not different between the two experiments, but heart rate was significantly decreased ($p \leq 0.01$) by an average of 6 beats•min^{-1} at rest and 9 beats•min^{-1} during exercise (Table 1).

Mean thermoregulatory data are presented in Table 1. Esophageal temperature increased more during exercise with PYR treatment than during the control experiment

($p \leq 0.05$). Whole body sweating rate during exercise was increased during PYR by an average of 15.7% ($p = 0.08$). In order to determine whether whole body sweating rate during exercise was significantly increased, the sweating data from the two other male volunteers were included in the statistical analysis to increase the number of observations in regard to the sweating data. When the whole body sweating rate data were analysed for seven subjects, there was a significantly increased whole body sweating rate during exercise which averaged 12.7% ($p = 0.01$). Forearm blood flow, as measured by venous occlusion plethysmography, was not different between the control and PYR experiments. However, skin blood flow, as estimated from laser doppler velocimetry, was significantly decreased both at rest and during exercise with PYR treatment ($p \leq 0.05$).

Fig. 1 shows the mean SkBF as estimated from LDF, T_{es} and $\bar{T}_{sk}$ as a function of exercise time in the two experiments. The reduction in SkBF averaged 36.8% (Fig 1). Fig. 2 shows mean forearm blood flow as a function of exercise time in the two experiments. There was no significant difference in FBF between the control and pyridostigmine esperiments (Table 1). FBF was not analyzed as a thermoregulatory effector because the FBF measurement included muscle blood flow which was probably increasing while skin blood flow was decreasing. In this situation, FBF was not proportionally related to T_{es}, so the FBF to T_{es} relationship was not analyzed. The T_{es} thresholds for onset of cutaneous vasodilation and slopes of the SkBF to T_{es} relationship generated from the linear regression equations describing the exercise transient data for each experiment are shown in Table 2. The SkBF to T_{es} relationship during the exercise transient phase is shown for the subject who had the largest threshold change in Fig. 3. The T_{es} threshold for onset of cutaneous vasodilation was significantly increased ($p \leq 0.01$) during PYR compared to control experiments. The slope of

the SkBF to T_a relationship was reduced by 35% during PYR as compared to the control experiment, but the slopes in the two experiments were not significantly different ($p=0.22$) due to the highly variable responses of the individuals.

DISCUSSION

Since FBF included not only skin blood flow, but also blood flow to the muscle, subcutaneous fat and bone, we interpreted the lack of difference between the two conditions as the PYR treatment having opposite effects in at least two of the compartments in which blood flow could be changing. Since LDF measures blood flow only to an approximate depth of 1 mm (30), it seems reasonable to conclude that only skin blood flow was measured with this method (18,19,30,36). Since skin blood flow was decreased with PYR treatment at rest, then muscle (or some other tissue) blood flow may have increased to give an unchanged FBF. During exercise, FBF did increase in both experiments which reflected the increasing skin blood flow in response to increasing internal temperature (Fig. 2) However, FBF during exercise was not significantly different between experiments.

Pyridostigmine treatment may result in excessive stimulation of cholinergic receptors as shown by reports that pyridostigmine treatment increases muscarinic and nicotinic cholinergic neurotransmission in humans (35). In fact, the principal clinical use of reversible anti-ChE agents is to prolong nicotinic cholinergic neurotransmission at the skeletal neuromuscular junction in myasthenia gravis (35). Accumulation of ACh should occur at sites of neurotransmission where ACh is a neurotransmitter. If ACh is the neurotransmitter involved in reflex cutaneous vasodilation, acetylcholinesterase inhibition should increase the magnitude of reflex cutaneous vasodilation. This experimental approach is quite different from increasing ACh in the arterial blood as was done in the experiments of Roddie et al. (26) because ACh

should not accumulate at the vascular endothelium and prompt vasodilation through the action of endothelium-derived relaxing factor.

Pyridostigmine binds reversibly to AChE and the reversible nature of this bond makes the drug an attractive prophylactic agent against a class of other anti-ChE agents used as pesticides or chemical warfare agents which bind irreversibly to AChE. In these experiments, we wanted to determine how acute treatment with the reversible anti-AChE, pyridostigmine affected thermoregulation in humans during exercise. Pyridostigmine effectively inhibited acetylcholinesterase in these subjects and we presumed that there was greater accumulation of free ACh at cholinergic receptor sites (34,35,38). That presumption appeared justified as heart rate was significantly decreased during rest and exercise (Table 1) and whole body sweating rate was increased with PYR treatment. These changes can be explained by increased accumulation of ACh at the cholinergic receptor sites of each organ. The effects of PYR treatment on heart rate and whole body sweating rate have been reported before (11,35).

The observation that acute pyridostigmine treatment resulted in decreased skin blood flow in humans during rest and exercise is new and may provide additional information about the control of skin blood flow. Since the sweating and heart rate responses in these subjects after acute PYR treatment were consistent with an increased accumulation of ACh or a direct agonistic action of PYR on cholinergic receptors on the sweat gland and heart, it could logically be expected that this treatment should also result in increased ACh accumulation at those cholinergic neuronal sites which affect skin blood flow. If cholinergic innervation of vasodilatory fibers to the cutaneous vessels is the major component of the control of skin blood flow, it could then be expected that PYR treatment would cause increased skin blood

flow. The observation that cutaneous blood flow was reduced requires that an alternative explanation be formulated.

Rowell (27.28,29) has pointed out that existence of specific vasodilatory nerves is still not proven, and active cutaneous vasodilation could be through mediation of the cholinergic neurotransmission to the sweat glands. The data from the current study appear to be inconsistent with cholinergic activation of reflex cutaneous vasodilation. Increased accumulation of ACh at the paravertebral sympathetic ganglion could be one site where enhanced cholinergic receptor stimulation could promote increased vasoconstrictor tone. This possibility is consistent with the observation of decreased skin blood flow at rest and during exercise. It is also consistent with the observation that the T_{es} threshold for onset of cutaneous vasodilation was increased with PYR treatment as a shift in the threshold has been classically interpreted as a central modulation of the thermoregulatory system (4). If increased accumulation of ACh at the pre-ganglionic cholinergic sympathetic receptors resulted from PYR treatment, then the relative vasoconstriction observed would be a centrally mediated event.

Pyridostigmine itself has been reported to affect nicotinic cholinergic receptors in an in vitro preparation of the rat forebrain (12), although it is not known whether pyridostigmine directly affects these receptors in humans. It is thought that the drug is impeded by the blood-brain barrier (35), but there is a possibility that the drug will cross the blood-brain barrier if in sufficiently high concentration. In any case, the accumulation of ACh resulting from PYR treatment could cross the blood-brain barrier to act on the brain.

The acute treatment of volunteers with the anticholinesterase, pyridostigmine bromide, increased the esophageal temperature threshold for the onset of cutaneous vasodilation by

approximately 0.2°C (Fig. 3). An increased T_{sk} threshold for onset of a thermoregulatory effector has generally been interpreted as a central modulation in the thermoregulatory system (4), although it is possible that a change in threshold might be the result of a alteration at the site of the effector (periphery). Pyridostigmine bromide should not cross the blood-brain barrier (35), at least in the low concentration used in these experiments. However, accumulated ACh, which results from PYR treatment, could be acting centrally. But, ACh would be limited by its diffusion from sites of neurotransmission. There is also a possibility that there is increased vasomotor tone caused by accumulation of ACh at the autonomic ganglion which in turn may result in the increased T_{sk} threshold for onset of cutaneous vasodilation with PYR treatment through increased sympathetic release of norepinephrine. In this case, the modification in thermoregulatory control would still be in the nervous system, although not in the brain. Another possible explanation for the increased T_{sk} threshold for cutaneous vasodilation during PYR is the local effect on skin blood flow by the slightly reduced mean skin temperature. The average mean skin temperature was 0.2-0.3°C less during PYR treatment, but such an insignificant difference in mean skin temperature should not affect the T_{sk} threshold for onset of cutaneous vasodilation (18). It is also possible that bradycardia caused by PYR treatment initiated a baroreflex which reduced cutaneous blood flow.

In summary, this investigation reported that acute administration of pyridostigmine bromide in humans resulted in decreased heart rate and skin blood flow at rest and during exercise and an increased sweating rate during exercise. The observed heart rate and sweating rate responses to the drug were reported before (11,35), but the reduction in skin blood flow is a new observation. Some possible mechanisms for the reduced skin blood flow which was observed with PYR treatment may be: 1) increased vasomotor tone in the skin resulting from

ACh accumulation at the autonomic ganglion; 2) excessive accumulation of ACh at cholinergic vasodilatory fibers to cause autoinhibition (31); 3) bradycardia resulting from PYR treatment may promote relative vasoconstriction through a baroreflex; or 4) the accumulation of ACh to a great enough extent to diffuse into the brain to cause a central effect. Clearly, future studies, perhaps involving the measurement of sympathetic nervous activity to the skin during reflex cutaneous vasodilation, are required to determine the mechanism causing the observed reduction in cutaneous blood flow during pyridostigmine treatment.

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TABLE 1. Mean (±SD) resting and exercise (25th min) temperature data for control and following pyridostigmine (PYR) treatment.

	REST		EXERCISE	
	Control	PYR	Control	PYR
Esophageal temperature (°C)	36.58 (0.3)	36.58 (0.3)	37.29 (0.3)	37.44 (0.3)
Change in T_{es} (°C)			0.7 (0.2)	0.9 (0.1)*
Mean weighted skin temperature (°C	33.7 (0.4)	33.5 (0.3)	33.3 (0.7)	33.0 (0.6)
Whole body sweating rate ($g \cdot min^{-1}$)			10.2 (2.4)	11.8 (2.3)‡
Forearm blood flow ($ml \cdot 100\ ml^{-1} \cdot min^{-1}$)	2.5 (0.8)	1.8 (0.7)	15.4 (5.9)	13.9 (5.9)
Skin blood flow (mV)	31.8 (13)	18.0 (8)**	114.4 (37)	84.8 (41)**
Metabolic rate ($W \cdot m^{-2}$)	49.8 (6)	49.8 (6)	327.9 (56)	337.1 (65)
Heart rate ($beats \cdot min^{-1}$)	63 (8)	56 (6)*	134 (10)	125 (9)*

* Different from control ($p \leq 0.01$)
‡ $p=0.08$. When an additional two subjects were added, $p=0.01$
** Different from control ($p \leq 0.05$)

TABLE 2. T_{es} thresholds for initiation of cutaneous vasodilation and slopes (SkBF to T_{es}) of the individual linear regression equations generated from each subject's transient response to exercise.

Subject	T_{es} Threshold (°C)		Slope ($mV \cdot °C^{-1}$)	
	Control	PYR	Control	PYR
1	36.71	36.84	119.0	81.1
2	36.61	36.81	298.7	133.3
3	36.59	36.86	172.5	64.9
4	37.11	37.32	139.4	153.7
5	37.19	37.30	44.7	74.7
X	36.84	37.03*	154.9	101.5
(S.D.)	0.29	0.26	93.1	39.4

T_{es}, esophageal temperature

*Significantly different from control ($p \leq 0.01$)

FIGURE LEGENDS

Figure 1. Mean (±SD) skin blood flow (SkBF;1a), esophageal temperature (1b) and mean skin temperature (1c) as a function of exercise time during the control and pyridostigmine experiments. Resting data are presented at time = -5 min. *All data different between treatments.

Figure 2. Mean (±SD) forearm blood flow as a function of exercise time for the control and pyridostigmine experiments. Resting data are presented at time = -5 min.

Figure 3. Individual skin blood flow data from a single subject (#3) plotted as a function of esophageal temperature data from the exercise transient period during the control and pyridostigmine experiments.

Fig. 1

FIGURE 1a

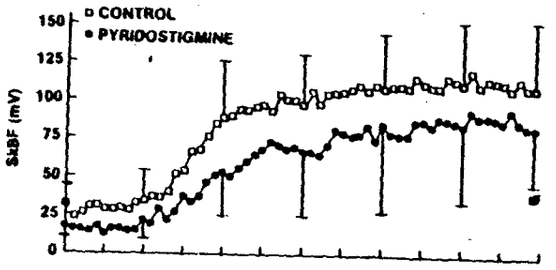


FIGURE 1b

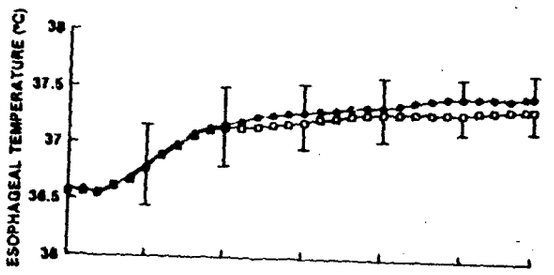


FIGURE 1c

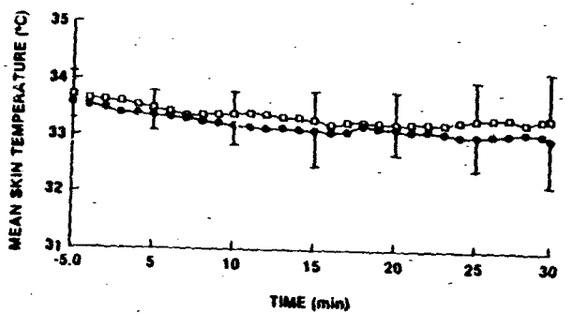


Fig. 2

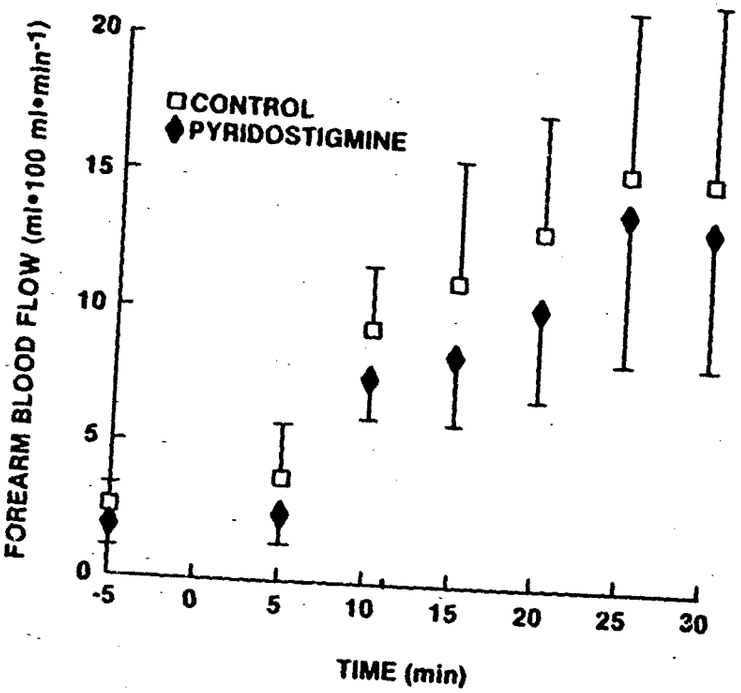
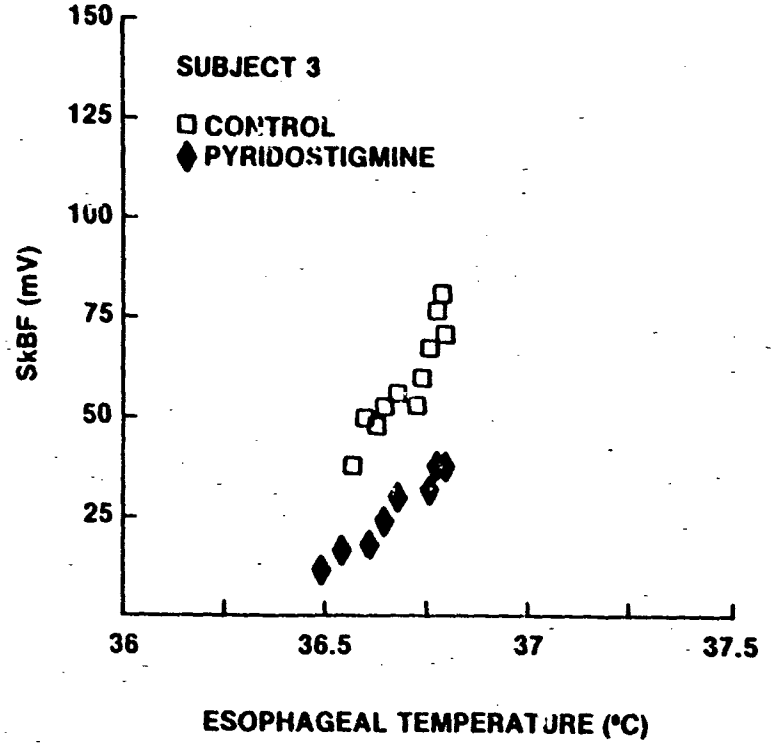


Fig. 3


150
125
100
75
50
25
SkBF (mV)
SUBJECT 3
CONTROL
PYRIDOSTIGMINE
36
36.5
37
37.5
ESOPHAGEAL TEMPERATURE (°C)